

Physicochemical Properties Of ^{131}I -Rutin Under Acidic Labeling Condition As A Radiolabeled Compound For The Diagnosis Of Cancer

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Abstract: Radiolabeled rutin (^{131}I -rutin) has potential use in future diagnosis of cancer. The physicochemical characteristics of ^{131}I -rutin are essential in regard to its distribution and accumulation in the body. Therefore, to ensure successful delivery of a labeled compound, it is necessary to evaluate its physicochemical properties. The labeling of ^{131}I -rutin was accomplished using the oxidation method assisted by Chloramine-T under acidic conditions. Radiochemical purity value was measured by thin-layer chromatography (TLC-SG F₂₅₄) using 100% methanol as the mobile phase. The electrical charge of ^{131}I -rutin was determined by the paper electrophoresis. Moreover, the lipophilicity (P) was evaluated by the partition coefficient in the organic-water liquid. Plasma protein binding was determined in vitro by precipitation method using a 5% trichloroacetic acid (TCA) solution. The results showed that ^{131}I -rutin has a neutral charge and a lipophilicity value (Log P) of 0.395 ± 0.203 (hydrophilic), and was bound to human blood plasma proteins with a percentage of $69.36\% \pm 1.88\%$.

INTRODUCTION

Rutin is a flavonol glycoside found in fruits, vegetables, and plant-derived beverages such as tea and wine. Rutin has significant scavenging properties on oxidizing species. Therefore, it shows several pharmacological activities including anti-inflammatory, antitumor, antibacterial, antiviral, and anticarcinogenic effects. Rutin offers an advantage over myricetin, quercetagenin, and other flavonoids, which can act as the prooxidant agents and catalyze oxygen radical production. Rutin also possesses favorable safety profile over some pharmaceutical agents, for example aglycones (1). Antioxidant compounds could prevent cancer progression because they can eliminate free radical substances. BHA (butylated hydroxy aniline) and BHT (butylated hydroxytoluene) are synthetic antioxidants that are widely used in various food products, but also known to have some side effects, such as cause liver damage. Recently, people prefer to consume antioxidant-based products from natural sources such as flavonoids, vitamin C, and beta carotene (2). Therefore, antioxidant compounds from the flavonoid group such as Rutin can potentially be

developed as a labeled compound or radiopharmaceutical to detect cancer.

Cancer is one of the main health problems as a leading causes of early deaths and disabilities within society. Globocan data indicates that in 2018 there were 18.1 million new cases of cancers with 9.6 million deaths. Furthermore, 1 in 5 men and 1 in 6 women in the world experience cancer(3). The incidence of cancer in Indonesia is 136.2 / 100,000 populations, ranks 8th in Southeast Asia, and 23rd in Asia (4). Therefore, efforts are needed to detect cancer quickly and accurately. Currently, various cancer diagnosis methods have been developed, such as laboratory tests, imaging tests, and biopsies. There are several methods exploited for imaging, namely Mammograms, Computerized Tomography Scan (CT Scan), Magnetic Resonance Imaging (MRI), Nuclear Scan, Bone Scan, PET Scan, Ultrasound, and X-rays (5). However, these diagnostic techniques possess some limitations, such as mammographic diagnostic techniques cannot distinguish between benign breast lesions and cancer. In addition, according to Notosiswoyo (2004), although the use of MRI as a cancer detection is very effective, there are side effects (especially

the latent effect) considering the magnetic field strength is quite high. In addition, the cost of MRI examination is relatively expensive.

Therefore, a nuclear cancer diagnosis method can be an alternative for detecting cancer in an effective manner. Radiopharmaceuticals are pharmaceutical formulations consisting of radioactive substances (radioisotopes and molecules labeled with radioisotopes), which are intended for use either in diagnosis or therapy (6). As a radiopharmaceutical product, it must go through a labeling procedure using radionuclides with nuclear characteristics that are sufficient for the intended use in nuclear medicine.

Radionuclides emitting gamma radiation that can be used as tracers include ^{99m}Tc and ^{131}I . In this study, ^{131}I was chosen because it has a long half-life (8 days 57.6 minutes) and has a high energy of 364 keV for gamma radiation and 606 keV for beta radiation. ^{131}I is the first radionuclide used in vivo for hyperthyroidism and different forms of treatment for thyroid cancer. Also, ^{131}I is used for radioactive labeling of several substances used for radiodiagnosis and radiotherapy procedures. Therefore, the ^{131}I -Rutin is expected to be an ideal alternative as a cancer radiodiagnosis agent(7).

The flavonoid iodination was previously reported by B.N Park. et.al (2001) using Iodine-123 (8), iodination of apigenin by Seyitoglu, et.al (2009) (9), and rutin iodination by Hee et. al (10) and sriyani, et.al (11). For clinical applications, either diagnosis or therapy, the radiopharmaceuticals must meet the requirement stated by the Food and Drug Monitoring Agency, the Ministry of Health, and the Nuclear Energy Supervisory Agency. One of the ideal radiopharmaceutical requirements is it must has a radiochemical purity more than 90%. The stability of drugs was affected by five conditions: chemistry, physics, microbiology, toxicology, and therapeutics. These aspects should be investigated because of possible product degradation that may alter the safety and efficacy of the drugs. Another requirement that must be fulfilled is that the radioisotopes must be bound stably to the compound. Also, the labeling element and the radioisotopes must not cause changes in the physical and chemical characteristics of the labeled compound. This research aimed to determine the physicochemical characteristics of ^{131}I -rutin, including pH, electrical charges, plasma proteins binding, and lipophilicity.

EXPERIMENTAL SECTION

Material

Sodium-iodide[^{131}I], Rutin (quercetin-3-rutinoside) (Aldrich), human serum plasma, dimethyl sulfoxide (Merck), Phosphate Buffer Saline (PBS), acetic acid 50% (E. Merck), Chloramine-T Hydrate (Sigma Aldrich), sodium metabisulfite (E. Merck), aquabidest sterile (IPHA), Chloroform (E. Merck), methanol (E. Merck), n-octanol (E. Merck), trichloroacetic acid (E. Merck), saline solution (IPHA), TLC-SG F₂₅₄, Whatman no 1.

Instrumentation

Analytical balance (Mettler Toledo), incubator (Mettmert), Dose Calibrator, TLC Scanner, Oven (Mettmert), Vortex Mixer.

Labeling of ^{131}I -rutin

As much as 200 μL Rutin solution (10 mg / mL DMSO) was placed into a metal-free vial. Subsequently, 100 μL of the acetic acid solution and 50 μL Na- ^{131}I (3.7 MBq) were added. The mixture was then incubated and shaken at 1000 rpm for 5 minutes at room temperature. Then, 20 mL chloramine-T (1 mg/mL) was added to the vial. Initial pH was checked using a pH indicator. After ^{131}I -Rutin labeling was achieved, 530 μL of sterile aquabidest and 20 μL Na₂S₂O₅ (1 mg/mL) were added to the vial to obtain a final volume of 1 mL. The final pH was tested using a pH indicator, and also the initial radiochemical activity was measured using a dose calibrator.

The purification step was carried out through the Liquid-Liquid Extraction (ECC) method using water and chloroform. The ^{131}I -rutin was placed into a separating funnel then the extraction process was carried out using 3 x 1 mL chloroform. The mixture shaken until the two phases separated. The top layer is the water while the bottom layer is the chloroform. Chloroform and water layers were separated into different vials and then measured using a dose calibrator to determine the final radioactivity.

The compounds contained in the aqueous phase will be used for the next testing. Before use, radiochemical purity was determined by thin-layer chromatography method with TLC-SG F₂₅₄ as a stationary phase and methanol as an eluent, and electrophoresis with Whatman 1 as a stationary phase and a phosphate buffer of 0.2N pH of 7.4 as an electrolyte.

Determination of Electric Charge

The ^{131}I -Rutin electrical charge was determined by the paper electrophoresis method using Whatman paper no.1 (1 cm x 37cm) as a stationary phase (marked from -18 to 18) and a phosphate buffer of 0.2 N pH 7.4 as an electrolyte solution in the vessel electrophoresis. Then, a few drops of compound solution were placed to the center of the electrophoresis paper. Electrophoresis was carried out for 1 hour at a voltage of 350 volts. The electrophoresis paper was then dried in an oven and cut every 1 cm and counted with SCA (12).

Determination of Lipophilicity

In a 5 mL test tube containing 500 μL NaCl physiological solution (NaCl fraction) and 500 μL n-octanol solution (octanol fraction), 50 μL of ^{131}I -Rutin solution were added. Then, the mixture was shaken using a vortex mixer for 1 minute and centrifuged for 15 minutes. Then, the n-octanol and NaCl fractions were separated by five (5) μL each and dropped on Whatman chromatography paper no.1. After that, the chromatography paper count using SCA. The experiment was repeated three (3) times. The amount of lipophilicity (Log P) was calculated using equation (1).

$$\text{Lipophilicity} = \frac{\text{Count on octanol fraction}}{\text{count on NaCl fraction}} \quad (1)$$

Determination of Plasma Protein Binding

A total of 50 μL ^{131}I -Rutin was added to a centrifuge tube containing 500 μL human blood plasma. The mixture was shaken with a vortex mixer for 1 minute and incubated at 37°C for 15 minutes. Then, 1 mL of 5% TCA was added to the mixture, stirred with a vortex mixer, centrifuged for 15 minutes, and the precipitate and supernatant were separated. The precipitate was washed using NaCl with a comparable volume. After that, the separation of the supernatant and sediment was carried out again. Each fraction was calculated by the SCA. The experiment was repeated three (3) times. The amount of plasma protein binding calculated using equation (2)

$$\% \text{ protein binding} = \frac{\text{Counts on precipitate}}{\text{counts (precipitate+filtrate)}} \quad (2)$$

RESULTS AND DISCUSSION

Radioiodination of rutin occurred through electrophilic substitution in which hydrogen atoms attached to electron-rich systems, such as aromatic rings. The atoms will be replaced with electrophiles (I^+ at H_2OI^+), forming carbon-iodine

covalent bonds in rutin flavonol rings. The position of hydrogen that can be replaced by I^+ was influenced by the surrounding functional groups (13). Electrophile I^+ will replace hydrogen atoms in carbon atoms numbers 8 in the rutin flavonol ring. The proposed structure of the ^{131}I -Rutin is seen in Figure 1.

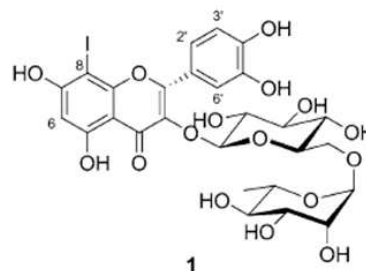


Figure 1. Proposed structure of iodine-rutin (10)

In this study, the labeling of rutin with radioisotope ^{131}I takes place in an acidic conditions (pH 4-5) with the addition of 50% acetic acid. It aims to minimize the formation of unidentified side products and only one product observed (10). Several labeling methods can be used for radioiodination, but in this research, we only used the chloramine-T method. Labeling rutin with Iodine-131 is a reduction-oxidation (redox) reaction. Chloramine-T is used to oxidize iodine. Oxidized iodine derivatives are readily reactive and therefore relevant for both the iodination step and for side reactions of hormone biosynthesis. These reactive oxidation products include I^+ , I_2 , OI^+ , HOI , IO_3^- , H_5IO_6 , and $\text{H}_3\text{IO}_6^{2-}$ (14). Rutin that added to chloramine-T was shaken using a 1000 rpm vortex shaker for 5 minutes to increase kinetic energy so that the collision event was more effective, and therefore, the I-oxidation reaction will be optimized. The formula used in labeling rutin with radioisotope ^{131}I can be seen in table 1.

Chloramine-T is an oxidizing agent with a fast reaction rate so that reducing agents (e.g., sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$)) were needed to stop the reactions (15). The reducing agent changed I^+ back to I^- in the equilibrium reaction and prevents over-labeling and oxidative damage to rutin compounds as target molecules. The amount of sodium metabisulfite added is five (5) times the amount of chloramine-T used so that the oxidation process was expected to stop completely, and there is no oxidative damage to routine compounds. Ideally, for every one molecule of oxidant two molecules of reductant are needed to stop the reaction (16). However,

this also has the potential to increase the number of impurities formed.

Table 1. Formulation of ^{131}I -Rutin

Composition	Amount
Rutin Solution	200 μL
CH_3COOH 50%	100 μL
$\text{Na-}^{131}\text{I}$	50 μL
Chloramine-T	20 μL
Incubation	5 min
Aquabidest	530 μL
$\text{Na}_2\text{S}_2\text{O}_5$	100 μL
Initial pH	4
Final pH	3
Final volume	1000 μL

The Liquid-Liquid Extraction (ECC) method with chloroform as an organic phase, will isolate the I_2 impurities formed during the ^{131}I -Rutin labeling process. I_2 impurities can be formed from the reaction between I^+ and I^- . Chloroform will attract impurities I_2 , while the ^{131}I -rutin remains in the NaCl fraction. The differences between Rutin and ^{131}I -rutin solubilities are because of halogen bond. The halogens act as Lewis base and will significantly increased solubility of molecular halogens in water upon addition of halide ion(17). Halogen bonding may also reduce lipophilicity and enhance solubility(18). The extraction was carried out with 3 x 1mL chloroform. This step will dissolve the I_2 in ^{131}I -rutin compound in chloroform. The physicochemical properties of ^{131}I -rutin is presented in table 2.

Table 2. Physicochemical result of ^{131}I -rutin

No	Result
pH	4
Clarity	Clear
Radiochemical purity	$93.44 \pm 2.59\%$
Radiolabeling efficiency	85.70 ± 6.19
Electrical charge	Neutral
Lipophilicity (log P)	0.44 ± 0.16
Protein binding	$66.00 \pm 2.56 \%$

The determination of ^{131}I -Rutin radiochemical purity was carried out using the Thin Layer Chromatography (TLC) method. TLC-SG F₂₅₄ was used as a stationary phase and 100% methanol as a mobile phase (11). This research was conducted to determine the presence of imourities that can accumulate in organs and cause adverse effects during in vivo imaging. The presence of radiochemical impurities in a radiopharmaceutical reduces the quality of

imaging due to the high background radiation from the surrounding tissues and blood, and gives unnecessary radiation burden to the patient (19). The radiochemical impurities in radioiodination can be I_2 and I^- . The I_2 impurity was minimum because most of the I_2 has been separated during the liquid-liquid extraction process. In this study, the radiochemical purity of the ^{131}I -Rutin was $93.44 \pm 2.59\%$ (Figure 2). This value meets the requirements outlined in the British Pharmacopeia and the United States of Pharmacopeia (>90%) (11).

The ^{131}I -Rutin labeling efficiency was determined from the radioactivity after and before the liquid-liquid extraction process. The high labeling efficiency indicates that the labeling process is increasingly optimal. In this study, ^{131}I -Rutin labeling efficiency was obtained at $85.70\% \pm 6.19\%$. The labeling efficiency is possible at equilibrium (99.99%). Excellent radiolabeling efficiencies are possible with radioiodide for iodine exchange reactions, even if some carrier iodide is present (20).

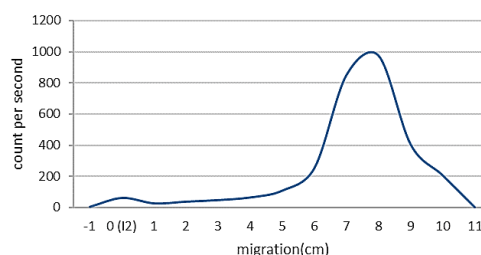


Figure. 2 Radiochromatogram of ^{131}I -Rutin

The charge of a molecule will affect the distribution in the body. Negatively charged particles can be rapidly opsonized and massively cleared by the fixed macrophages (21). The ^{131}I -Rutin electric charge was determined by the paper electrophoresis method. A negatively charged compound will move towards the anode (positive pole) whereas a positively charged compound moves towards the cathode (negative pole), and a neutral compound will remain at zero. In this study, a Whatman No.1 paper was used as the stationary phase and a phosphate buffer solution of 0.2 N pH 7.4 as an electrolyte solution. The phosphate buffer solution serve as a conduction bridge between the two electrodes to allow the electricity to occur, stabilizes the supporting medium, and affected the speed of the compound because the high ionic strength in the buffer will increase the heat so that the flow of electricity will be maximum. The results indicated that the compound has a neutral charge at $R_f = 0$ (Fig. 3).

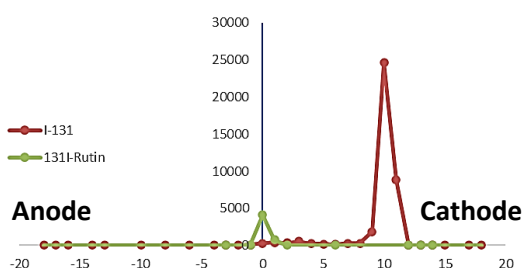


Figure 3. The paper electrophoresis results of Na^{131}I and ^{131}I -rutin

Passive permeation of drugs through the biological cell membranes is strongly dependent on the molecule's physicochemical properties including its lipophilicity (22). Lipophilicity plays an important role in the absorption, distribution, and elimination of a drug. The polar compounds will easily be soluble in water and fast clearance through the kidneys, and often highly polar compounds, cannot penetrate the blood-brain barrier (BBB). The neutral, lipophilic molecules could enter the brain through the BBB (25). A compound is lipophilic if the partition coefficient value is high. Lipophilic compounds will more easily penetrate lipid membranes because of their fat solubility. The partition coefficient will associate with the drug distribution after absorption, and how fast it is metabolized and eliminated. Elimination of a drug is often caused by drug toxicity which has to be estimated at the earliest possible stages of drug discovery, even before synthesis (24). One of the standards used for medicinal compounds is the rule of five identified in Lipinski's rule of five. A compound is likely to be cell membrane permeable and easily absorbed by the body based on the following criteria: molecular weight of the compound lower than 500 Da; logP lower than 5; the number of hydrogen bond donors (usually the number of hydroxyl and amine groups in a drug molecule) lower than 5; the number of groups that can accept hydrogen atoms to form hydrogen bonds (estimated by the number of oxygen and nitrogen atoms) lower than 10 (22). One of these rules states that drug compounds should have a log P value below five. From the results, the lipophilicity value (log P) of ^{131}I -Rutin was $0,44 \pm 0,16$, indicating that the ^{131}I -Rutin was hydrophilic. Therefore, we expect that ^{131}I -Rutin is non-toxic and rapidly excreted by the kidneys to minimize radiation exposure to the body.

According to Widyasari, et.al, plasma protein binding of a radiopharmaceutical will

affect its biodistribution and uptake in tissue or organ target and clearance from plasma (plasma clearance) (25). High binding to plasma proteins will influence pharmacokinetic profile as well as reduce blood clearance. Since the free fraction of molecules will penetrate biological barriers and reach the therapeutic target, the high plasma binding properties may reduce the therapeutic effect (26). The determination of plasma protein binding is carried out using the in-vitro deposition method with a solution of trichloroacetic acid (TCA) 5% as a precipitating agent. TCA 5%, which is a strong acid that can damage the structure of the secondary and tertiary protein so that the protein will precipitate. After all, it is amphoteric because it has two different charges in one molecule (zwitterion). At a certain pH, when it reaches the isoelectric point where the amount of total protein content equal to zero, the protein can undergo protein precipitation due to very low solubility. Centrifugation utilizing centrifugal force resulting two phases in the solution: the precipitate and supernatant. The results demonstrated that the plasma binding value of ^{131}I -Rutin was $66.00\% \pm 2.56\%$. This value suggest that ^{131}I -Rutin will be excreted more slowly from the body. Excretion time is also influenced by the biological half-life of ^{131}I -Rutin, thus allowing sufficient time for imaging with gamma cameras in nuclear medicine.

CONCLUSION

Radiolabeling of rutin with iodine-131 was carried out using the Chloramine-T method. It was observed that the physicochemical properties of ^{131}I -rutin are as follows: a clear yellow solution (free of particles) with pH 3, neutral charge, and $\text{Log P} = 0.44 \pm 0.16$. The percentage of ^{131}I -Rutin bound to plasma protein was $66.00\% \pm 2.56\%$, with a radiochemical purity of $93.44 \pm 2.59\%$. Based on these results, it is necessary to further perform the preclinical studies both in-vitro and in-vivo.

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